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Sprenger, Herman

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Chapter 7

Antiretroviral therapy only partially reverses the hypercoagulable state and reduced fibrinolytic potential of HIV-1 infection

Herman G. Sprenger, Wouter F.W. Bierman, Ton Lisman, André B. Mulder, Karina Meijer, and Vladimir Y.I.G. Tichelaar

Submitted

SUMMARY

The incidence of venous thrombosis in untreated HIV-1 infected patients is increased. This is consistent with increased levels of procoagulant proteases and decreased levels of anticoagulant ones, partially caused by HIV infection itself and partially by HIV-associated comorbidity. The role of combination antiretroviral therapy (cART) on the risk of venous thrombosis and coagulation levels is on the long term unclear. Therefore, we studied the effect of one year of cART on the coagulation system. We performed a prospective, longitudinal cohort study in antiretroviral-naïve patients who were followed up for 48 weeks after initiation of cART. Tests for coagulation markers, thrombin generation and fibrinolytic potential were performed before and after start of cART. Forty men with HIV-1 infection were included. At baseline, levels of hs-CRP, D-dimer, FVIII and von Willebrand factor were significantly elevated, as well as the endogenous thrombin potential (ETP), peak thrombin level and velocity index from the thrombin generation assay, compared to healthy controls. Also, a clear increase in the clot lysis time was found. After 48 weeks of cART, levels of D-dimer, FVIII, von Willebrand factor and ETP (at 24 weeks) showed a significant decline, and levels of protein S were significantly increased. However, the haemostatic balance did not completely normalize. Clot lysis time remained significantly increased in patients compared to healthy controls at 24 weeks. Our results suggest that HIV-1 infected patients are in a procoagulant and hypofibrinolytic state before starting cART, which only partially improves after 48 weeks of cART.

INTRODUCTION

The incidence of venous thrombosis (VT) is increased in HIV-1 infected patients, with a risk 1.3 to 10 times greater than in HIV-negative control populations [1-5]. This increased risk for VT is at least partly mediated by the presence of increased levels of procoagulant factors such as factor VIII (FVIII), von Willebrand factor (VWF), fibrinogen, and tissue factor (TF) [6-11] and decreased levels of anticoagulant factors such as AT, protein C and (free) protein S [9,10,12-15]. Increased levels of plasminogen-activator inhibitor type 1 (PAI-1) leading to impairment of endogenous fibrinolysis can also contribute to this increased risk [16]. Most studies found a relationship with advanced HIV disease and with high HIV-RNA levels [5-7,9,10,12,15-17], showing that (chronic) inflammation and hypercoagulability share common pathways [18]. Indeed, HIV infection is characterized by systemic immune activation and inflammation [19] and increased levels of interleukin (IL)-6 and high-sensitivity C-reactive protein (hs-CRP) have been reported [20-23]. Although the use of combination antiretroviral therapy (cART) decreases most markers of immune activation and inflammation, many studies have shown that immune activation persists, even after many years of cART-induced viral suppression [24-26]. Certain inflammatory markers are of prognostic significance regarding all-cause mortality, non-AIDS related death and cardiovascular disease (CVD) [22,23,27]. Other markers of persistent immune dysfunction, such as a decreased ratio of CD4+ to CD8+ T cells (CD4/CD8 ratio) despite cART, have also been shown to be an independent predictor of serious non-AIDS events and mortality [28].

The effect of cART on hypercoagulability is less clear. Persistent immune activation may, despite cART, also contribute to a procoagulant state and increased risk of VT. Some studies showed that treatment of HIV infection improves coagulation abnormalities [6-8,29], but certain antiretroviral drugs, especially protease inhibitors (PI), might be risk factors themselves [1,3,4,10,30].

Due to a lack of prospective studies with a longer follow-up time, still little is known about the influence of cART on the haemostatic system in HIV-infected patients. Therefore, we performed a longitudinal study in antiretroviral-naïve HIV-1 infected patients to assess the effect of cART on the coagulation system during the first 48 weeks.

METHODS

Study participants

We conducted a single-centre, prospective cohort study of consecutive, antiretroviral-naïve, HIV-1 infected subjects visiting the outpatient clinic or staying on the hospital ward and for whom the treating physician had decided that cART should be started. Subjects 18 years of age or older were eligible. Exclusion criteria were HIV-2 infection, pregnancy, use of oral contraception and not being able to understand the Dutch or English language. The ethics committee of the hospital approved

the study protocol. All participants provided written informed consent. There was no commercial sponsorship.

Study design and blood sampling

At baseline (t=0), before cART was started, data were collected from all subjects, including demographics, general medical history, risk factors for venous and arterial thrombosis, (family) history regarding venous and arterial thrombosis, medication use, and smoking. Blood samples were taken at baseline and at 4, 12, 24 and 48 weeks after start of cART. Blood was drawn by venous puncture from the antecubital vein. For all coagulation tests, blood was collected into a 5-mL citrated Vacutainer, anticoagulated with 1:10 volume of 0.109 mol/L trisodium citrate. Platelet-poor plasma (PPP) was prepared by centrifugation at 2500 x g for 15 minutes, aliquoted and immediately frozen at -80 degrees Celsius and analysed later after rapidly thawing at 37 degrees Celsius.

Assays

We measured fibrinogen with Dade® Thrombin Reagent from Siemens, and D-dimer with Tina-quant reagent from Roche Diagnostics (Mannheim, Germany). A one-stage FVIII activity (FVIII:C) assay was performed with APTT reagents (Dade® Actin® FS Reagent) and factor deficient plasmas, obtained from Siemens and measured on a CA-7000® system (Sysmex Corporation, Siemens). VWF was tested by ristocetin cofactor (VWF:RCo) activity measured with Von Willebrand Reagent (lyophilized stabilized platelets and ristocetin) from Siemens in an optical aggregometer from Chrono-Log Corp (Haverton, PA, USA). Total protein S antigen levels were measured with an enzyme-linked immunosorbent assay (ELISA) with reagents obtained from DAKO (Glostrup, Denmark). Free protein S antigen levels were assessed by ELISA after precipitation of protein S bound to C4-binding protein with 3.75% PEG 6000. Activity of protein C (Berichrom Protein C, Dade Behring, Liederbach, Germany) and AT (Coatest, Chromogenix, Mölndal, Sweden) were assessed by chromogenic substrate assays. Post hoc, we decided to determine thrombin generation by calibrated automated thrombinography and clot lysistime as global tests of coagulation and fibrinolysis, and to measure tissue factor pathway inhibitor (TFPI) levels. For these tests, specimens of 16 patients were available from both baseline and 24 weeks. Thrombin generation testing was performed with the fluorometric method using PPP in the presence and absence of thrombomodulin as described previously [31]. Thrombin generation variables analysed were endogenous thrombin potential (ETP), peak thrombin generation, lag time (time needed for thrombin concentration to reach $1/6^{th}$ of the peak concentration) and velocity index (slope between the end of lag time and peak thrombin generation). Furthermore, a normalized thrombomodulin sensitivity ratio (TM-SR) was determined by dividing the ETP in the presence of thrombomodulin divided by the ETP in the absence of thrombomodulin of an individual, by the ETP in the presence of thrombomodulin divided by the ETP in the absence of thrombomodulin of pooled normal plasma. A TM-SR >1 reflects a decreased anticoagulant response to thrombomodulin in comparison with pooled normal plasma. Clot lysis time was assessed by using an in-house, plasma-based clot lysis assay, measuring the changes in plasma turbidity during tissue-factor induced clot formation and

subsequent lysis by exogenous tissue plasminogen activator as previously described [32]. Free TFPI antigen levels were determined using an ELISA as described previously [33].

Furthermore, CD4+ and CD8+ T-cell count (cells/mm^3) were measured by flow cytometry, and HIV-1 RNA by polymerase chain reaction (lower limit of detection 40 copies/mL; Abbott m2000 Real-Time HIV-1, Abbott Laboratories, Wiesbaden, Germany).

Outcomes

Primary outcomes were mean differences in levels of pro- and anticoagulants measured before the start of cART and 48 weeks after start of cART. The following markers were measured: D-dimer, FVIII, VWF, antithrombin (AT), protein C, protein S, free protein S. We also measured high-sensitive C-reactive protein (hs-CRP), fibrinogen, CD4+ T-cell counts and HIV RNA levels at the same time points during the study. Secondary outcomes were mean differences in parameters of thrombin generation by calibrated automated thrombography (CAT), clot lysis time, and in tissue factor-pathway inhibitor (TFPI) levels, between t=0 (before start of cART) and 24 weeks after start of cART. These outcomes are secondary and post-hoc determined, therefore we had not enough plasma available of all patients. We only could analyse the secondary outcomes in 16 random patients, of whom we had enough plasma at t=0 and 24 weeks. The selection of these patients was utterly random (due to the presence of leftover citrated plasma). Finally, we performed post hoc subgroup analyses on the outcome variables FVIII, VWF, free protein S, D-dimer, and ETP with thrombomodulin, for the following subgroups: type of cART regimen (PI-containing cART versus non-PI-containing cART), patients with a viral load above versus below the median of the group before start of cART, patients with a detectable viral load versus undetectable viral load at t=48w, patients with a CD4/CD8 ratio above versus below the median of the group before start of cART, patients with a CD4/CD8 ratio above 1 versus below 1 at t=48w and patients who had a chronic comorbidity, i.e. Kaposi sarcoma, non-Hodgkin lymphoma, chronic hepatitis B virus infection and chronic hepatitis C virus infection during the study versus patients without such a chronic co-morbidity. Finally, a sensitivity analysis for the primary and secondary outcomes was performed by excluding patients with a malignancy, acute or chronic infections, or anticoagulant use.

For the individual coagulation and anticoagulant factor assays we used the normal values of the laboratory as reference values. A control group of 30 healthy non-matched volunteers, working at our laboratory yielded reference values for the thrombin generation test and the clot lysis time test, and TFPI.

Statistical analysis

Data of baseline characteristics are presented as means with standard deviation (SD) or as medians with range for continuous data (stated between parenthesis), and as counts (n) and percentages (%) for categorical data. To correct for missing data in the analyses, we used multiple imputation techniques (method=auto, number of imputations=10). Consequently, for the primary and secondary outcomes,

we presented means with standard errors of the mean (SEM) (Table 2 and 3), and calculated a p for trend as a general estimate of the effect of cART over time on the outcomes. Second, we calculated a difference of means between baseline levels and levels at 48 weeks of treatment with cART (t=48w) for primary outcomes, and a difference of the mean between baseline levels and levels at 6 months of treatment with cART for secondary outcomes. For this method, we used a design of mixed models for repeated measures with a Bonferroni correction when testing for statistical significant differences. For dichotomous data, we used Cochran's Q test for k-related samples to calculate a p for trend.

For each subgroup analysis, we used general linear models for repeated measures to look for interactions between the within-groups variable of time (on cART) and between-groups factors on defined outcomes. For definition of these factors and outcomes, see the paragraph Outcomes above here. All statistical analyses were performed using IBM SPSS, version 20 (IBM Corp., Armonk, NY, USA).

RESULTS

Participants

Consecutive subjects were enrolled from July 2009 through May 2011. Patient characteristics at baseline are shown in Table 1. Forty men were included, of which 87.5% were men who have sex with men. All subjects started with their cART according to the prevailing guidelines at the time of the study, which recommended starting cART in patients with a CD4+ T-cell count <350 cells/mm^3. Median CD4+ T-cell count was 262 cells/mm^3 (range 10-670). Ten patients (25%) had a CD4+ T-cell count of ≤200 cells/mm^3. Of the 40 participants, one had a VT and two an arterial thrombotic event (2.5 and 5% respectively) in their medical history.

Table 1. Baseline characteristics of 40 patients

	n or mean	(%) or SD
Male sex	40	(100)
Age (years)	40.7	15.3
Ethnicity		
African origin	5	(12.5)
Caucasian origin	35	(87.5)
HIV transmission		
Homosexual	35	(87.5)
Heterosexual	4	(10)

Table 1. *Continued*

	n or mean	(%) or SD
Time since diagnosis (months) (median, range)	10.5	(0-100)
HIV-1 RNA (copies/mL) (median, range)	87,100	(22,000-7,600,000)
CD4 count (cells/mm^3) (median, range)	262	(10-670)
CD4/CD8 ratio (median, range)	0.24	(0.05-0.58)
VTE in history	1	(2.5)[1]
VTE in first-degree relative	6	(15)
ATE ever	2	(5)[2]
BMI (kg/m^2)	23.5	3.2
Hypertension	7	(17.5)
Antihypertensive drugs	6	(15)
Hypercholesterolemia	2	(5)
Lipid-lowering drugs	4	(10)
Diabetes mellitus type 2	1	(2.5)
Oral antidiabetic drugs	1	(2.5)
Smoking		
Former	10	(25)
Current	17	(42.5)
Packyears	19	14
Anticoagulation therapy	3	(7.5)[3]
Malignancy	2	(5)[4]
Chronic hepatitis B virus infection	2	(5)
Chronic hepatitis C virus infection	1	(2.5)
cART at start		
TDF/FTC/EFV	27	(67.5)
TDF/FTC + DRV/r	6	(15)
TDF/FTC +ATV/r	4	(10)
TDF/FTC + RAL	1	(2.5)
ZDV/3TC + LPV/r	1	(2.5)
ZDV/3TC + EFV	1	(2.5)
Protease inhibitor use	11	(27.5)
cART switch	8	(20)

VTE, venous thrombotic event; ATE, arterial thrombotic event; BMI, body mass index; cART, combination antiretroviral therapy; TDF, tenofovir; FTC, emtricitabine; EFV, efavirenz; DRV/r, ritonavir boosted darunavir; ATV/r, ritonavir boosted atazanavir; RAL, raltegravir; ZDV, zidovudine; 3TC, lamivudine; LPV/r, ritonavir boosted lopinavir.

[1] First and recurrent pulmonary embolism in the same patient, diagnoses before HIV diagnosis.

[2] Two participants with acute coronary syndrome, diagnosed before HIV diagnosis.

[3] One subject on vitamin K antagonist, two on low dose aspirin.

[4] Two participants with Kaposi's sarcoma, one also with non-Hodgkin lymphoma.

Table 2. Mean levels of inflammatory and coagulation parameters of 40 HIV-1 positive patients over time

	t=0	t=4w	t=12w	t=24w	t=48w	*P* value for trend	Mean Diff.*	95%CI
hs-CRP (0-3.0mg/L)†	5.0 (1.4)	4.6 (1.1)	4.2 (1.0)	6.6 (3.0)	2.7 (0.4)	n.s.	-2.3	-6.1 to 1.5
fibrinogen (1.7-4.0g/L)	3.1 (0.11)	2.8 (0.11)	2.8 (0.11)	2.9 (0.16)	2.8 (0.08)	<0.05	-0.3	-0.6 to 0.01
D-dimer (0-499ng/ml)	564 (125)	468 (120)	306 (83)	251 (60)	156 (42)	<0.01	-378	-623 to -133
FVIII:C (50-150IU/dL)	222 (11)	188 (9)	180 (10)	165 (9)	161 (8)	<0.001	-61	-89 to -32
vWF-ag (50-150IU/dL)	211 (15)	176 (12)	161 (10)	143 (9)	132 (8)	<0.001	-79	-113 to -46
AT (80-120IU/dL)	102 (2)	109 (4)	109 (2)	109 (1)	111 (2)	<0.05	8	2 to 15
Protein S-ag (65-150IU/dL)	93 (3)	98 (2)	101 (2)	101 (3)	101 (3)	<0.05	7	-1 to 16
Protein C-act (65-150IU/dL)	100 (4)	102 (3)	103 (3)	107 (3)	104 (3)	n.s.	4	-6 to 14
Free protein S (60-140IU/dL)	76 (5)	82 (5)	93 (5)	89 (4)	93 (4)	<0.001	16	5 to 27
CD4 (500-1300 cells per mm^3)	261 (200)	328 (210)	384 (220)	414 (250)	466 (240)	<0.001	205	150 to 260
HIV-1 RNA (copies per mL)	480,398 (177,278)	20,151 (14,426)	106 (22)	26 (11)	10 (5)	<0.001	-480,388	-939,705 to -21,071
Undectable (<40 copies per mL), n (%)	0	2 (5)	14 (35)	30 (77)	34 (85)	<0.001	-	-

* Between t=0 and t=48w; † Reference values of the hospital laboratory. n.s.: not significant (*P*>0.05)

Data are means with standard error of the mean; t=0, baseline; w=weeks; hs-CRP=high-sensitivity C-reactive protein; FVIII:C=factor VIII activity; vWF=von Willebrand Factor; ag=antigen; act=activity.

Effectiveness of antiretroviral therapy

The vast majority of the participants had a rapid decline of HIV-1 RNA. Twenty-four and forty-eight weeks after starting cART, HIV-1 RNA levels were undetectable in 30 of 39 (77%), and 34 of 40 participants (85%) respectively. Mean CD4+ T cell count was 466 cells/mm^3 (SEM 240) after 48 weeks of treatment, a statistically significant increase of 205 cells/mm^3 (95% confidence interval [CI], 150 to 260) compared to CD4+ T cell levels at baseline (Table 2).

Markers of coagulation

Before the start of cART, levels of FVIII, VWF and D-dimer were clearly elevated (mean levels 222 IU/dL [SEM 11], 211 IU/dL [SEM 15] and 564 ng/mL [SEM 125], respectively) compared to the reference values of the laboratory (Table 2). Although mean free protein S levels (76 IU/dL [SEM 5]) were within reference range, they were below the lower limit of the reference in 11 subjects (28%). D-dimer levels were above the upper limit of normal (500 ng/mL) in 16 patients (40%). FVIII, VWF and D-dimer levels steadily decreased over time during treatment, reaching lowest levels at 48 weeks after start of cART (difference of the means between t=0 and t=48w: -61 IU/dL (95% CI: -89 to -32) for FVIII, -79 IU/dL (95% CI: -113 to -46) for VWF and -378 ng/ml (95% CI: -623 to -133) for D-dimer). However, FVIII and VWF levels did not reach reference levels in 22 (56%) and 11 (28%) subjects, respectively. Mean free protein S levels had increased with 16 IU/dL (95% CI: 5 to 27) after 48 weeks on cART, with one patient still having a level below the reference range.

Clot lysis time, thrombin generation parameters and plasma levels of TFPI

These tests were performed in 16 subjects for whom paired plasma specimens were available (Table 3). Availability was random, patient characteristics for these 16 subjects and the whole group were similar (data not shown). The mean clot lysis time in HIV-1 positive patients was significantly increased compared to controls (81 minutes [SEM 8] versus 51 [SEM 7]). After 6 months on cART, the mean clot lysis time decreased slightly with 9 minutes (95% CI: -22 to 4 minutes), but remained significantly increased compared to controls. All thrombin generation parameters that included thrombomodulin in the model showed significantly increased levels in patients at baseline compared to controls (Table 3). Most striking changes were observed in the ETP with thrombomodulin, with a mean of 682 nM*min (SEM 50) in patients compared to 510 nM*min (SEM 259) in controls. After 6 months of cART, ETP with thrombomodulin levels decreased significantly with 126 nM*min (95% CI: -247 to -5) to 556 nM*min [SEM 50], a comparable level to that of the controls. Calculation of a TM-SR demonstrates thrombomodulin resistance in HIV-1 infected patients (TM-SR patients median 1.3 [range 0.8-2.0], controls median 1.1 [range 0.3-1.7]; $P<0.05$). Plasma levels of TFPI at baseline were significantly higher in patients than in controls (mean level 199% [SEM 14] versus 78% [SEM 31], $P<0.05$), and did not decrease after 6 months of cART.

Subgroup analyses

We observed significant interaction terms ($P<0.05$) for the outcomes of FVIII and VWF with time, for patients that had a viral load below the median at baseline and patients with a viral load above the

median at baseline. While baseline levels of FVIII and VWF did not differ between subgroups, both FVIII and VWF levels decreased less quickly and less profoundly over time and were significantly higher after 48 weeks of cART in patients with HIV RNA *below* the median at baseline (mean FVIII level 182 IU/dL, mean VWF level 152 IU/dL), compared to the group with HIV RNA levels *above* the median at baseline (mean FVIII level 141 IU/dL, mean VWF level 112 IU/dL) (both *P*<0.05).

Table 3. Clot lysis time, TFPI levels and thrombin generation parameters of patients and controls

		Cases				
	Controls	**t=0**	**t=24w**	**Mean Diff.†**	**95%CI**	***P* value for trend**
CLT (min)	51 (7)	81 (8)*	71 (8)*	-9	-22 to 4	n.s.
ETP (nM.min)	945 (267)	1050 (47)	989 (47)	-61	-197 to 76	n.s.
ETP+TM (nM.min)	510 (259)	682 (50)*	556 (50)	-126	-247 to -5	<0.05
Peak (nM)	188 (50)	235 (12)*	222 (12)*	-13	-48 to 21	n.s.
Peak+TM (nM)	131 (53)	184 (13)*	157 (13)	-27	-59 to 5	n.s.
Velindex (nM/min)	79 (31)	92 (7)	87 (7)	-4	-25 to 16	n.s.
Velindex+TM (nM/min)	68 (29)	90 (8)*	77 (8)	-13	-30 to 5	n.s.
Lagtime (min)	1.5 (0.21)	2.3 (0.09)*	2.3 (0.9)*	-0.016	-0.27 to 0.24	n.s.
Lagtime+TM (min)	1.5 (0.19)	2.1 (0.07)*	2.0 (0.07)*	-0.016	-0.22 to 0.19	n.s.
TFPI (%)	78 (31)	199 (14)*	194 (14)*	-5	-35 to 24	n.s.

Data are means with standard deviation (controls) or standard error of the mean (cases)
w=weeks; CLT=clot lysis time; ETP=endogenous thrombin potential; TM=thrombomodulin; Peak=maximum thrombin concentration generated; Velindex=velocity index; TFPI=tissue factor pathway inhibitor.
* *P*<0.05 compared to controls; † between t=0 and t=24w; n.s.: not significant (*P*>0.05)

Two significant interactions (*P*<0.05) were found regarding levels of free protein S. Patients with a viral load above median at baseline had lower levels of free protein S at baseline (mean 68 IU/dL), compared to patients with a viral load below median at baseline (mean 85 IU/dL, mean difference 17 IU/dL [95% CI: -0.2 to 34]). A similar difference in free protein S levels at baseline was also observed between patients with a CD4/CD8 ratio below the median versus those with a CD4/CD8 ratio above the median at baseline (mean levels 68 versus 84 IU/dL, respectively; mean difference 16 IU/dL [95%CI: -1 to 33]).

Finally, we observed a significant different trend over time regarding levels of FVIII and VWF between patients who had a CD4/CD8 ratio <1 at t=48w and patients with a (normalized) CD4/CD8 ratio >1 at t=48w (n=5). Over time, all levels of FVIII and VWF were significantly (*P*<0.05) lower in patients with a low CD4/CD8 ratio than in patients with a high CD4/CD8 ratio (mean difference in FVIII: 51 IU/dL [95%CI: 5-100] and in VWF: 57 IU/dL [95%CI: 9-105]).

Further subgroup analyses, as stated in the methods section, did not result in significant differences in trends over time between groups for the other outcomes. Our sensitivity analysis (exclusion of patients

with malignancy, acute or chronic infections, or anticoagulant use) had no influence on the analysis of our primary or secondary outcomes (data not shown). The subgroup effects as described above, were partially comorbidity driven, as we lost statistical significance for 3 of 4 above described differences after adjustment, except for the difference in FVIII levels at 48 weeks between patients with a baseline HIV RNA level below or above the median.

DISCUSSION

We showed that patients with an HIV-1 infection are in a procoagulant and hypofibrinolytic state before the start of cART. This is based on abnormally high levels of FVIII, VWF, D-dimer, decreased free protein S levels, increased thrombin generation parameters (endogenous thrombin potential, peak thrombin and velocity index) and elongated clot lysis time. Secondly, we showed that after starting cART, these parameters partially normalized, but the haemostatic balance remained slightly procoagulant and hypofibrinolytic, marked by persistently increased levels of FVIII, increased clot lysis time and a significantly higher peak thrombin parameter after at least 24 weeks on cART.

Improvement of markers of coagulation and endothelial activation, but not reaching reference levels, has also been demonstrated in other studies [6-8,29]. This is in analogy with studies that showed that markers of HIV-1 associated immune activation decrease after start of cART, but do not reach normal levels in HIV-1 infected patients, even after long-term treatment with successful suppression of HIV replication [24,34-37]. However, the participants that kept abnormal values of coagulation parameters at 48 weeks of follow-up were not typically those with a detectable HIV-1 RNA level at 48 weeks. Moreover, FVIII and VWF levels dropped more and faster in the group with a baseline viral load *above* the median, leading to a significant difference in FVIII and VWF levels between these subgroups after 48 weeks of cART. This might mean that patients that with a higher viral burden at baseline responded better to cART regarding levels of FVIII, VWF and free protein S as outcomes. Second, patients with a normalized CD4/CD8 ratio (>1) at t=48w, indicating better immune competence, had significantly higher levels of FVIII and VWF during the whole year than patients with a lower ratio. These somewhat counterintuitive findings might be partially explained by the presence of comorbidity in these patients, as after exclusion of patients with comorbidity the differences in levels of FVIII and VWF between patients with a CD4/CD8 ratio <1 and those with a ratio >1 at t=48w became smaller and lost statistical significance.

Our findings are in accordance with some other studies showing a significantly lower total thrombin generation in treated HIV-1 infected patients compared with untreated patients [10,38]. In contrast to our study, two other studies reported a significantly *lower* total thrombin generation in untreated *and* treated HIV-1 infected patients compared to non-infected controls [29,39]. Since the thrombin generation test is an overall test of coagulation reflecting pro-and anticoagulant reactions, our findings of elevated procoagulant and decreased anticoagulant factors at baseline as well as the

improvement of these factors during cART, are consistent with the results of the thrombin generation test we demonstrated.

We found a statistically significant prolonged lag time in the thrombin generation test in our patients that was not influenced by cART. In line with this finding, we showed that TFPI, an important inhibitor of initiation of coagulation and determinant of the lag time [40], was highly up regulated.

Our white, male patients kept an impaired plasma fibrinolytic potential during treatment. This is associated with an increased risk of venous and arterial thrombosis [41,42]. A cross-sectional study among black South African patients [43], showed that HIV-1 infected participants had a significantly longer clot lysis time than HIV-negative participants, but no information was given about the treatment status. Levels of PAI-1 are highly increased in HIV-1 infected patients [16] and remained significantly elevated after 48 weeks of cART compared to HIV-uninfected controls [44], thus offering a potential explanation for a prolonged hypofibrinolytic state, as PAI-1 is an important determinant of the clot lysis time [45]. A recent study found that high levels of PAI-1 in HIV-1 infected patients receiving cART were independently associated with a first myocardial infarction [46].

Our study has several strengths and limitations. Strengths of our study are the prospective design; the longer follow-up time compared to most previously reported studies (12 versus 6 months), and the homogeneity of its participants. The restricted number of patients and the lack of female participants form limitations. In addition, due to missing plasma samples we were not able to perform post-hoc testing in all participants and at all moments of follow-up. Furthermore, because of the sample size of the study and the still quite short follow-up time, we had too few patient-years of follow-up for clinically relevant vascular events to occur.

In conclusion, white male HIV-1 infected patients are in a procoagulant and hypofibrinolytic state before start of antiretroviral treatment, and some slightly remain so, in spite of significant improvement of the haemostatic balance caused by cART. This is best explained by persistent activation or dysfunction of the endothelium, pictured by our findings of still high levels of FVIII and TFPI, and an increased clot lysis time. Unfortunately, we were not able to fully characterize the individual patients subject to this phenomenon, although regain of immune competence together with comorbidity might partially explain these observations.

More studies are needed to determine the possible pathophysiological pathways of the persistent activation of the endothelium, as well as to understand the clinical relevance of our findings. Regarding the latter, we are currently performing an observational study to investigate whether HIV-1 infected patients still have an increased risk of VT under long-term successful cART. Finally, other studies could look into the role of the thrombin generation and clot lysis time test in predicting venous and arterial thrombotic events in patients with HIV-1 infection.

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